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The Histone Variant H3.3 Regulates Gene Expression during Lytic Infection with Herpes Simplex Virus Type 1[▽]

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It has been proposed that incorporation of the histone variant H3.3 within actively transcribed regions of a genome helps to facilitate transcription. In this report we use lytic infection by herpes simplex virus type 1 (HSV-1) as a model to examine the temporal profile of histone H3 incorporation and to determine whether the variant histone H3.3 has a direct effect on transcription. We find that canonical H3.1 and variant H3.3 exhibit distinct temporal associations with the genome in cell lines expressing equal amounts of epitope-tagged H3 variants. At the earliest times examined after infection, the HSV-1 genome is incorporated into chromatin that predominantly contains the variant H3.3, whereas incorporation of canonical H3.1 occurs later in infection and is dependent on replication of the HSV-1 genome. Further, inhibition of H3.3 association, via reduced expression of the H3.3 chaperone HIRA, significantly reduces the levels of HSV-1 mRNA. These findings show that incorporation of H3.3 facilitates transcription, and they provide new evidence for a regulatory role of chromatin composition during HSV-1 acute infection.

Herpes simplex virus type 1 (HSV-1) establishes lytic infections within mucosal epithelial cells and latent infections within sensory neurons. Latent HSV-1 can reactivate and reinfect surrounding tissue. During latent infection the HSV-1 genome is largely compacted into inactive heterochromatin (6, 35). However, the latency-associated transcript gene is active during latency, and the latency-associated transcript promoter contains high levels of histone H3 methylated at lysine-4 (H3K4me) and low levels of H3K9me (22). During lytic infection the HSV-1 genome is also associated with histone proteins although the extent and regularity of the nucleosomal pattern are still unclear (10, 18). In contrast to latency, during acute infection histones associated with the various temporal classes of HSV-1 genes (immediate-early [IE], early, and late) contain "active" chromatin marks, such as H3K4me and histone H3 acetylated at lysine-9 and -14 (H3K9/K14ac) (13, 18).

Within all eukaryotes, nuclear DNA is associated with histone proteins in a protein/DNA complex called chromatin (20). Chromatin modulation plays a central role in the regulation of DNA processes, such as replication, transcription, and repair (2). Chromatin can be divided into two higher-order classes, the relatively open euchromatin, where most transcription occurs, and the more compact heterochromatin. Numerous mechanisms alter the structure of chromatin, including ATP-dependent chromatin remodeling, posttranslational modification of the histones, and substitution of the canonical histones with histone variants (7). Transcriptionally active euchromatin is associated with a number of active chromatin marks, such as acetylation on histone H3 (e.g., at H3 lysine-9 and lysine-14), while heterochromatin is enriched in "inactive" chromatin marks (e.g., H3 lysine-9 methylation) (30).

Recent studies on the histone variant H3.3 has shed new light on mechanisms that alter chromatin content and structure (for a review, see references 9 and 31). Canonical histones, such as H3.1, are expressed concomitantly with DNA synthesis, whereas histone variants are expressed throughout the cell cycle and appear to play specific roles in chromatin dynamics. The histone variant H3.3 is a highly conserved protein. For example, in mammals it contains only four amino acid substitutions compared to the major form of histone H3. H3.3 is associated with actively transcribed regions of the genome (1, 3), and activation of transcription leads to replacement of H3.1 with H3.3 over transcribed genes (14, 32). A further indication of the importance of H3.3 in transcription is that the pattern of H3.3 deposition correlates with sites of abundant RNA polymerase II and with histone modifications that are associated with gene activation, such as histone H3 acetylation and H3 K4 methylation (5, 16, 26). Indeed, H3.3 itself is enriched in posttranslational modifications that correlate with active chromatin (e.g., H3K9ac, K14ac, and K4me3 [K4 trimethylation]), whereas, the canonical histone H3.1 is enriched in modifications correlating with repressive chromatin, such as H3K9me (25). Thus, the transcriptional state of chromatin may be altered via exchange of the canonical histone H3.1 with histone variant H3.3.

We use HSV-1 lytic infection as a model to examine the regulatory role of chromatin (13, 18). The viral particle is devoid of histone proteins (8, 28, 29), and prior to infection the viral DNA is uncoated and is apparently "naïve" with respect to chromatin structure. It is thus important to investigate how the naked HSV-1 genome becomes initially incorporated into chromatin. In this study we determined whether H3.1 and H3.3 are differentially deposited during the early stages of lytic infection and whether incorporation of H3.3 leads to higher

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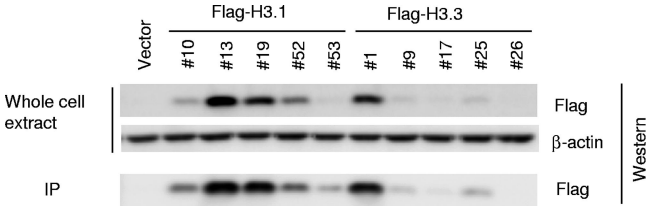

FIG. 1. HeLa cells stably expressing human Flag-tagged H3.1 and H3.3. Cell extracts were collected; inputs and Flag-immunoprecipitated samples were probed with monoclonal antibody to the Flag epitope. β-Actin was used as a loading control. All further experiments were carried out using clone 13 (Flag-H3.1) and clone 1 (Flag-H3.3). IP, immunoprecipitation; Western, Western blotting.

levels of transcription. Our results reveal that H3.1 and H3.3 have distinct incorporation profiles and different functions during HSV-1 lytic infection.

MATERIALS AND METHODS

Cell line and virus. HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics. Stable cell lines expressing either flag-tagged H3.1 or flag-tagged H3.3 were created in HeLa cells. The cells were selected with 2.5 μg/ml puromycin. The F strain of HSV-1 was used in all experiments. Cells were infected at multiplicities of infection (MOIs) of 1 to 5 as indicated on the figures. Samples were collected at 1, 3, 6, or 10 h postinfection.

siRNA treatment. Small interfering RNA (siRNA) against human HIRA was purchased from Dharmacon RNA Technologies. The target sequence for HIRA was as follows: 5′-GAAGGACUCUCGUCUCAUGUU-3′. siRNA was transfected into cells at a final concentration of 10 nM using Dharmafect 1 Transfection Reagent (Dharmacon RNA Technologies) according to the manufacturer's specifications. A control siRNA that targets the luciferase gene was also purchased from Dharmacon RNA Technologies, and the sequence was 5′-UAAG GCUAUGAAGAGAUAC-3′.

PAA treatment. Phosphonoacetic acid (PAA) was used to inhibit viral DNA polymerase. The final concentration of PAA was 400 μg/ml (12).

Nucleic acid analysis. Genomic DNA was isolated using a QIAamp DNA Blood Mini Kit (Qiagen), and RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. A reverse transcription reaction was carried out using TaqMan reverse transcription reagents (Applied Biosystems). Sybr Green reagent (Sigma) was used to determine the relative amount of double-stranded DNA products with an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Primer sequences and data analysis were described previously (18).

Chromatin immunoprecipitation (ChIP) assays. Cells mock infected and infected with the F strain of HSV-1 were processed as described previously (18). Briefly, cells were cross-linked with 1% formaldehyde for 15 min at room temperature. The cross-linking reaction was stopped by the addition of 0.125 M glycine. Cells were resuspended in lysis buffer (50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 0.1% Triton, pH 7.5). Cells were lysed, and the DNA was sheared by sonication into fragments between 200 and 500 bp. Prior to immunoprecipitation 1/10 of the extract was saved as the input. After immunoprecipitation the DNA was reverse cross-linked and subjected to real-time PCR analysis.

RESULTS

Incorporation of Flag-H3.1 and Flag-H3.3 into the HSV-1 genome. The HSV-1 genome is associated with histones during lytic infection (10, 18). We determined whether the H3 histone variant H3.3 is present and, if so, whether its incorporation affects gene expression. Currently, it is not possible to distinguish the endogenous H3 variants using immunological techniques because of the high degree of identity among their sequences; i.e., with only four amino acid differences between H3.1 and H3.3, making antibodies is difficult. Therefore, we

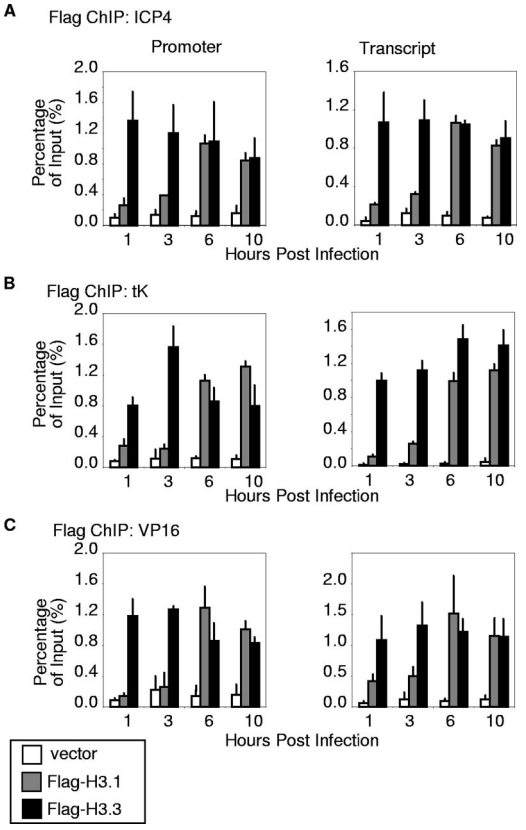

FIG. 2. Flag-tagged H3.1 and H3.3 are differentially incorporated into the HSV genome during acute infection. In all cases cells were infected at an MOI of 1 with HSV-1 and harvested at the indicated time postinfection. Data for the promoter and transcribed regions of the ICP0 (A), tK (B), and VP16 (C) genes are presented. The data are presented as percentages of input signals. All data represent the average of three independent experiments, and error bars represent 1 standard deviation of the data.

generated clonal HeLa cell lines stably expressing either Flag-tagged H3.1 (Flag-H3.1) or Flag-tagged H3.3 (Flag-H3.3) to serve as markers for endogenous H3.1 and H3.3; it has been shown that endogenous H3.1 is present at much higher levels than H3.3, as shown previously (24). Quantification of the Flag-tagged H3 proteins was determined by probing cell extracts with an antibody to the Flag epitope relative to equivalent amounts of β-actin. Cell lines were identified that expressed equal amounts of tagged H3.1 or H3.3 protein (Fig. 1). Subsequent experiments utilized Flag-H3.1 clone 13 and Flag-H3.3 clone 1 (Fig. 1). The matched cell lines have the advantage of ensuring that any differences observed in association of the epitope-tagged H3 variants with the HSV-1 genome are not attributable to differences in the amount of the variant or to differences in antibody affinity or specificity.

To determine the spatial and temporal distribution of the two H3 histones, ChIP experiments using Flag antibody were performed following HSV-1 infection. Real-time PCR signals using primers specific to the promoter and transcribed regions of the IE gene ICP4, the early gene thymidine kinase (tK), and the late gene VP16 were compared for the Flag vector control, Flag-H3.1, and Flag-H3.3 cell lines (Fig. 2). Flag-H3.3 is in-

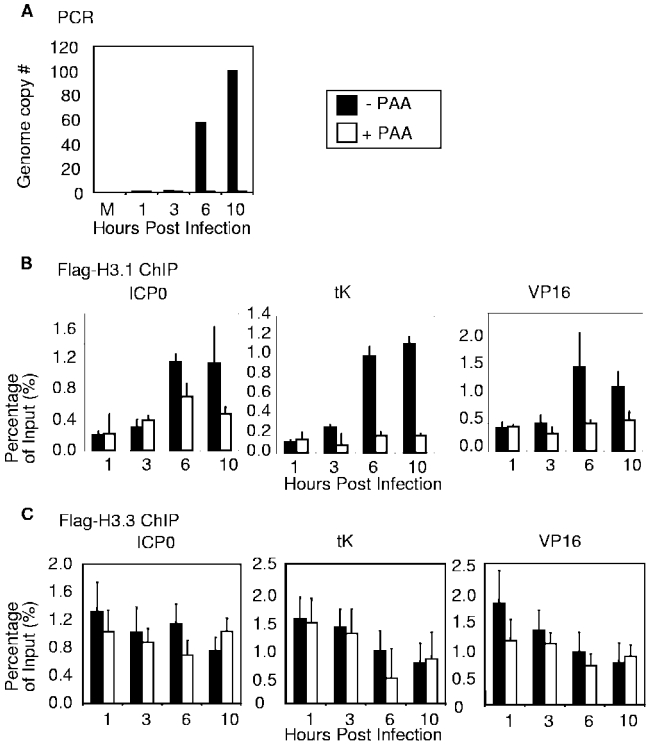

FIG. 3. Effect of PAA treatment on H3.1 and H3.3 incorporation. (A) The genome copy numbers for untreated and PAA-treated cells are shown. Genome copy number was quantified by real-time PCR and normalized to the number of cells. (B) The effect of PAA treatment on H3.1 incorporation in control cells and Flag-H3.1 cells. (C) PAA effect on H3.3 incorporation in control cells and Flag-H3.3 cells. All data represent the average of three independent experiments, and error bars represent 1 standard deviation of the data.

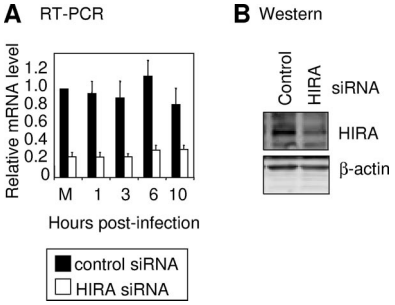

FIG. 4. siRNA directed to the human HIRA transcript reduces the level of HIRA mRNA and protein. (A) Effect of siRNA treatment on mRNA levels for HIRA. Cells were transfected twice with siRNA at 48 and 24 h prior to infection. mRNA was isolated at 1, 3, 6, and 10 h after infection at an MOI of 1 with HSV-1. Relative mRNA levels were determined by quantitative PCR and normalized to 28S rRNA. All data represent the average of three independent experiments, and error bars represent 1 standard deviation of the data. (B) Western blot analysis was performed with HIRA-specific antibody; β-actin was used as a loading control. RT-PCR, reverse transcription-PCR.

corporated into the promoter and transcribed regions of all genes tested at the earliest time sampled (1 h postinfection). The levels of H3.3 remain fairly constant throughout the time course, with only small fluctuations in the signal during the time course. Conversely, the levels of H3.1 incorporation are low at 1 and 3 h following infection and rise significantly at 6 and 10 h. This pattern occurs at all regions of the genome examined. The increase in H3.1 incorporation corresponds to the approximate time of HSV-1 replication, indicating that the incorporation of H3.1 is linked to genome replication, as analyzed further below. These general patterns of incorporation for H3.1 and H3.3 occur in the promoter and transcribed regions of all the HSV-1 genes examined, i.e., ICP4, tK, and VP16. These data suggest that immediately following infection, the uncoated HSV-1 genome is incorporated into chromatin predominately containing the histone variant H3.3, and then following replication at least a portion of newly replicated viral DNA is incorporated into H3.1 chromatin.

HSV-1 replication is required for incorporation of H3.1. To further examine the relationship between replication of the HSV-1 genome and incorporation of the histone H3.1, an inhibitor of the HSV-1 DNA polymerase, PAA, was used to inhibit DNA replication. PAA efficiently inhibited viral replication in this assay, as determined by quantification of the genome copy number of HSV-1 in the presence and absence of the inhibitor. In the absence of PAA, viral DNA levels increase in a typical fashion, dramatically rising between 6 and 10 h postinfection (Fig. 3A). In the presence of PAA, viral DNA levels are significantly reduced (Fig. 3A).

ChIP experiments were performed to determine whether PAA treatment affects H3.1 or H3.3 incorporation. Without PAA treatment H3.1 is incorporated within the coding regions of ICP0, tK, and VP16, as shown above, reaching a peak at approximately 10 h postinfection (Fig. 3B). However, addition of PAA leads to significantly reduced H3.1 incorporation relative to untreated cells, with levels two- to fourfold lower (Fig. 3B). The effect of PAA treatment on H3.3 incorporation at the same genes was examined. For H3.3 a slight but not significant decrease in signal in the PAA-treated cells was observed (Fig. 3C). These results strongly indicate that deposition of H3.1, but not H3.3, is coupled to viral replication. It should be noted that this result follows previous observations in a number of reports that the deposition of H3.1 is coupled to replication (33, 34).

HIRA siRNA decreases incorporation of H3.3 and HSV-1 gene expression. As described above, H3.3 deposition is independent of DNA replication and is linked to the histone chaperone HIRA (34). To investigate whether HIRA plays a role in the deposition of H3.3 during HSV-1 infection, siRNA knockdown was used to reduce HIRA expression. Cells treated with HIRA siRNA showed a significant decrease in HIRA mRNA (Fig. 4A) and lowered HIRA protein levels (Fig. 4B) compared to cells treated with control siRNA.

These cells were used to assess the effect of lowered HIRA on H3.3 incorporation into the HSV-1 genome. ChIP experiments using Flag antibody were performed on cells treated with control or HIRA siRNA. Cells bearing HIRA siRNA show decreased incorporation of H3.3 in the promoter and transcribed regions of the ICP0, tK, and VP16 genes (Fig. 5). The level of H3.3 is reduced two- to threefold in cells expressing lower HIRA levels compared to cells treated with control siRNA. The incomplete abolishment of H3.3 incorporation could be due to the fact that the levels of HIRA protein are reduced but not abolished (Fig. 4B) or because other chaper-

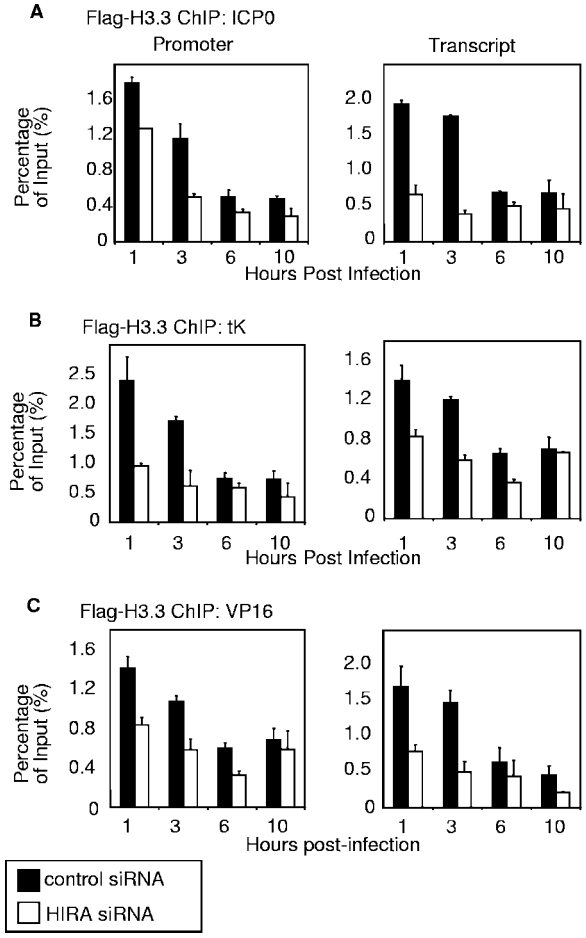

FIG. 5. HIRA knockdown lowers the incorporation of Flag-H3.3 within the HSV-1 genome. Flag-H3.3 incorporation was compared in control siRNA- and HIRA siRNA-treated cells stably expressing Flag-H3.3. Promoter and transcribed regions of ICP0 (A), tK (B), and VP16 (C) genes were examined by ChIP. Values are expressed as a percentage of input, and all data represent the average of three independent experiments; error bars represent 1 standard deviation of the data.

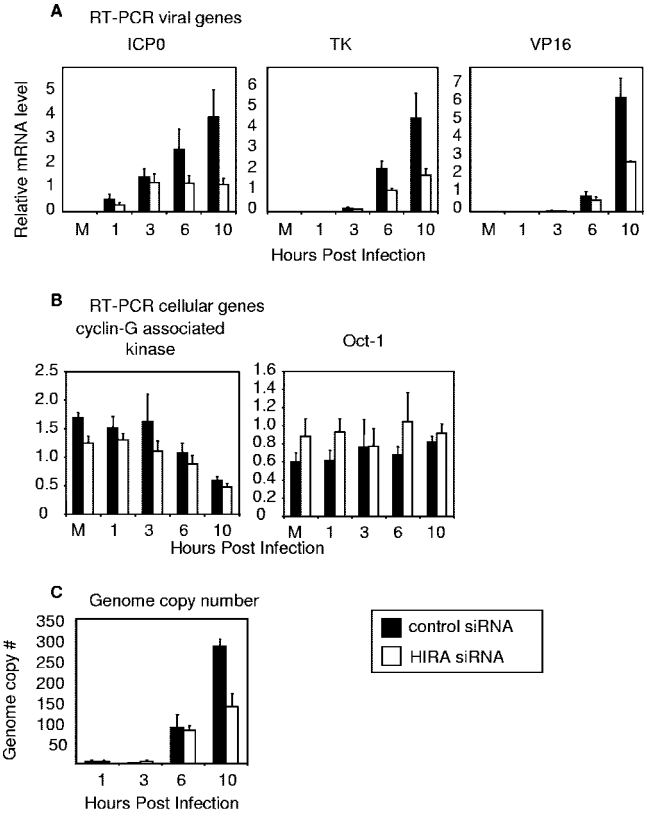

FIG. 6. Lowered H3.3 at HSV-1 genes leads to a decrease in gene expression and genome copy number. (A) HeLa cells were treated with control siRNA or HIRA siRNA. Cells were transfected twice with siRNAs at 48 and 24 h prior to infection. mRNA was isolated at 1, 3, 6, and 10 h after infection at an MOI of 1 with HSV-1. Relative mRNA levels for ICP0, tK, and VP16 (A) and cyclin-G-associated kinase (B) were determined by quantitative PCR and normalized to 28S rRNA. (C) Genome copy number was quantified by real-time PCR and normalized to the number of cells. All data represent the average of three independent experiments, and error bars represent 1 standard deviation of the data. RT-PCR, reverse transcription-PCR.

ones may be partially redundant with HIRA. Nonetheless, these results strongly indicate that HIRA is involved in the deposition of H3.3 within the HSV-1 genome. We examined the effect of the HIRA knockdown on H3.1 incorporation and observed no effect (data not shown).

In order to determine whether decreased H3.3 deposition on the HSV-1 genome correlates with altered gene expression, quantitative reverse transcription-PCR experiments were carried out on mRNA isolated from cells treated with control or HIRA siRNA. We examined the transcript levels of the ICP0, tK, and VP16 genes and found significantly reduced levels of HSV-1 gene expression (Fig. 6A). Specifically, ICP0, tK, and VP16 RNA levels were reduced two- to fourfold. These results indicate an important role of H3.3 in facilitating the transcription of HSV-1 genes. With respect to controls, we found no significant change in expression levels of two cellular genes in the presence of HIRA siRNA (Fig. 6B). We also tested a second HIRA siRNA sequence and obtained similar results (data not shown), indicating that the effect on HSV gene transcription in the HIRA knockdown is specific and not due to an off-target effect of the siRNA.

To determine the effect of this altered HSV-1 gene expression in the HIRA knockdown on viral replication, we examined HSV-1 genome copy number. We found that HSV-1 DNA levels are reduced twofold in the presence of HIRA siRNA (Fig. 6C). These results indicate that incorporation of H3.3 at HSV-1 genes is dependent on HIRA protein and is required for full expression and replication of the HSV-1 genome.

DISCUSSION

Recently the histone variant H3.3 has been the subject of intense investigation. H3.3 is deposited into chromatin independent of DNA replication, in contrast to replication-dependent H3.1. As an example, during sperm nucleus decondensation in *Drosophila*, H3.3 is deposited prior to the onset of DNA replication (19, 23). H3.3 is enriched at actively transcribed genes, and the deposition of H3.3 is triggered by induction of gene expression (5). H3.3 is differentially modified compared to H3.1 in that it is enriched in active chromatin marks com-

pared to H3.1, which is enriched in "inactive" chromatin marks (25), and H3.3 occupancy corresponds with RNA polymerase II sites (5, 26). All of these observations indicate a correlation between active transcription and H3.3, but a role of H3.3 in regulating gene expression remains to be fully characterized.

During lytic infection the HSV-1 genome becomes associated with histone proteins (10, 18), presenting a unique situation where a naïve genome, apparently completely devoid of chromatin, can be assessed for association of histone variants. In this report we examined incorporation of H3.1 and H3.3 into the HSV-1 genome at early times of infection using cell lines expressing similar amounts of Flag-tagged H3.1 or H3.3; thus, any observed differences between their incorporation will be due to intrinsic mechanisms of deposition rather than differences in their levels or antibody detection. Our results lead to the following conclusions regarding the role of H3 variants in HSV-1 acute infection: (i) H3.3 becomes associated with the genome earlier than H3.1 and before viral replication; (ii) reducing the levels of the H3.3 chaperone HIRA partially and selectively inhibits incorporation of H3.3, resulting in an overall decrease in gene expression and replication of the HSV-1 genome; (iii) incorporation of the canonical histone H3.1, but not H3.3, is dependent on the activity of the HSV-1 DNA polymerase and genome replication.

Our results suggest the existence of a mechanism for selective deposition of H3.3 onto the HSV-1 genome at early times of infection. While it is extremely difficult to assess the overall levels of endogenous H3.1 and H3.3 because their protein sequences are nearly identical, it is likely that the level of H3.1 is higher than that of H3.3 prior to infection; i.e., since the cells were not synchronized, there must be a distribution of the population through the cell cycle, and H3.1 is expressed at very high levels during S phase. Since the infection was carried out such that there were five times more infectious virus particles than cells, it is assumed that each cell is infected. Thus, at the onset of infection, it appears that H3.3 is predominantly deposited at all HSV-1 regions examined.

What mechanism could be responsible for the selective deposition of H3.3 at early infection times? It has previously been hypothesized that RNA polymerase may displace nucleosomes, leading to naked DNA at actively transcribed genes (27, 32). This naked DNA is then the template for replication-independent nucleosome assembly utilizing H3.3. However, our results indicate that "naked" HSV-1 DNA, devoid of histones upon entering the cell, is a sufficient template for nucleosome assembly utilizing the replication-independent pathway. Thus, active transcription appears not to be required for deposition of H3.3; for example, H3.3 is present in the promoter and transcribed regions of the VP16 gene, and this is many hours prior to transcription of VP16, which occurs concomitantly with DNA replication (13). This raises the possibility that there is a mechanism to initiate active recruitment of H3.3 to the HSV-1 genome. One possibility is through binding of VP16 to the IE genes, which in turn recruit a number of host transcription factors (10), and these could trigger H3.3 association, which is then spread throughout the genome.

Our results show that deposition of H3.3 is important for full expression of the HSV-1 genes. Reducing expression of HIRA using siRNA inhibited H3.3 incorporation throughout the genome, and this correlated with a significant decrease in the expression of HSV-1 genes and with reduced genome replication. This supports a hypothesis that H3.3 deposited at actively transcribed genes helps to increase the transcription of those genes. This view is supported by results using a different approach, which showed that expression of exogenous H3.3 led to increased gene expression of folate receptor and vascular endothelial growth factor D while expression of exogenous H3 reduced the expression of folate receptor and vascular endothelial growth factor D (15).

This increase in transcription caused by H3.3 deposition could be due to a number of factors. First, it is possible that deposition of H3.3 at actively transcribed genes could maintain this region of the genome in an open or transcriptionally primed state. This open state could allow for multiple rounds of transcription by allowing more polymerase to bind. Second, it has been demonstrated previously that H3.3 is enriched in active chromatin marks, such as K4 methylation and K9 and K14 acetylation (25). These active modifications may facilitate the recruitment of a host of cellular and viral transcription factors, which lead to an increase in transcription.

Our data indicate that the histone variant H3.3 plays an important role in HSV-1 lytic infection. While specific mechanisms need to be determined, it appears that HSV-1 harnesses the unique properties of H3.3 to achieve maximal transcription of its genes. Whether the deposition of H3.3 is a passive process, occurring primarily because the naked HSV-1 genome is an appropriate template for replication-independent nucleosome assembly, or an active process, occurring through a mechanism that may be enabled by the virus itself, has yet to be determined. However, we note that cells expressing an epitope-tagged H3.1 protein (which is available throughout the cell cycle unlike the endogenous protein, which is only available during the S phase) show incorporation only at later time points when the genome is being replicated. Thus, H3.1 deposition occurs at a specific time in the viral infection cycle, and this incorporation is not simply due to the availability of the protein. In fact, deposition of H3.1 appears to be coupled to DNA replication because when the cells are treated with PAA, a potent inhibitor of the viral DNA polymerase, H3.1 incorporation is reduced. We cannot eliminate the possibility that the decrease in H3.1 incorporation after PAA treatment might be due to the lack of true late gene transcription caused by the PAA treatment. True late genes are HSV genes that require DNA replication for their full transcription (4, 11, 17). It is possible that one of these genes might be required for H3.1 deposition; this question will require further study.

It has previously been reported that the HSV-1 genome is associated with histones and that these histones bear specific chromatin modifications (6, 10, 13, 18, 21, 22, 35). Here, we demonstrate for the first time that the HSV-1 genome is associated with a histone variant, H3.3, that has an important regulatory role for the virus and, thus, further elucidates the role of chromatin in the life cycle of HSV-1.

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